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Analysis of phosphatidylethanolamine, phosphatidylcholine, and plasmalogen molecular species in food lipids using an improved 2D high-performance liquid chromatography system

Ryosuke Takahashi[a,b], Makoto Nakaya[a,c,d], Miyako Kotaniguchi[a,c], Aiko Shojo[a,d], Shinichi Kitamura[a,*]

[a] Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai 599-8531, Japan
[b] YMC Co., Ltd., 284 Daigo-cho, Karasuma Nishiiru Gojo-dori, Shimogyo-ku, Kyoto 600-8106, Japan
[c] International Polysaccharide Engineering Inc., 1-2 Gakuen-cho, Naka-ku, Sakai 599-8570, Japan
[d] Department of Food and Nutrition Management Studies, Faculty of Human Development, Soai University, 4-4-1 Nanko-naka, Suminoe-ku, Osaka 559-0033, Japan

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ABSTRACT

Phospholipids are an important class of lipids in cell membranes and food. Several high-performance liquid chromatography (HPLC) methods have been developed to analyze phospholipids at the molecular species level. We developed a two-dimensional HPLC system with a charged aerosol detector and mass spectrometry (MS) to analyze phosphatidylethanolamine (PE), phosphatidylcholine (PC), and their plasmalogens (pls) extracted from food materials. Accordingly, the phospholipid molecular species can be analyzed in a single step despite using smaller samples. We confirmed that chromatogram peaks from soybean lecithin are mostly baseline separated, assigned, and quantified (24 molecular species for PE and 27 for PC). In addition, it was confirmed that chromatograms of lipids extracted from chicken breast meat include plasmalogen peaks. The PE fraction in lipids extracted from chicken breast meat contained 17 types of ethanolamine plasmalogens, corresponding to approximately 57% of the total by weight. The PC fraction contained only four choline plasmalogens, corresponding to approximately 11% of the total weight. The composition of the pls-PC molecular species differed from that of pls-PEs. The polyunsaturated fatty acids connected at the *sn*-2 positions of the pls-PEs consisted of 20.5% 20:4 fatty acid and were independent of the carbon chain at the *sn*-1 position. However, the 18:1 fatty acid at the *sn*-2 position was dependent on the carbon chain at the *sn*-1 position.

1. Introduction

Phospholipids are the major constituents of biological membranes and play an essential role in cell membrane function [1]. Phospholipids are also found in soybeans, egg yolks, and fish roe, and they are used in emulsifiers, dispersants, pharmaceuticals, and food supplements [2,3]. In the human diet, triacylglycerols (TAGs) are major fatty acid (FA) carriers; however, the bioavailability of some FAs, such as docosahexaenoic acid and arachidonic acid, can be more efficient in phospholipids than in TAGs [4,5]. Phosphatidylethanolamines (PEs) and phosphatidylcholines (PCs) are the main constituents of food phospholipids.

Plasmalogens are a unique subclass of phospholipids characterized by the presence of a vinyl-ether bond instead of an ester bond at the *sn*-1 position of the glycerol backbone [6,7]. Plasmalogen levels can be markedly increased by ingestion [8]. These compounds are particularly abundant in chicken breast meat and skin, as well as in bivalves, such as scallops. They have attracted considerable attention as sources of functional foods and supplements [9,10]. The vinyl-ether bond at the *sn*-1 position makes plasmalogens more susceptible to oxidative stress than the corresponding ester-bonded phospholipids [11]. Dietary plasmalogens have been proven to increase the relative ethanolamine plasmalogen (pls-PE) composition of erythrocyte membranes in rats, causing a decrease in the plasma cholesterol and phospholipid levels [12].

Reverse-phase high-performance liquid chromatography (RP-HPLC) is often used to study the molecular composition of phospholipids. RP-HPLC coupled with a mass spectrometer (MS) is a conventional tool for phospholipid identification and quantification [13–16]. Using the corresponding standard lipid samples, the targeted phospholipid molecular species can be precisely quantified [17–19]. Multiple standard lipid samples, corresponding to numerous molecular species, are required for global analysis.

* Corresponding author.
E-mail address: skita@bioinfo.osakafu-u.ac.jp (S. Kitamura).

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Two-dimensional HPLC (2D-HPLC) systems offer several advantages, including ease of automation, greater reproducibility, and more rapid analysis [20–24]. Current 2D-HPLC systems combining the hydrophilic interaction liquid chromatography (HILIC) mode and RP mode provide a powerful means of analyzing phospholipid molecular species [22–24]. The HILIC mode can be used for high phospholipids samples, such as egg, soya, and porcine brain. However, Lísa et al. showed that non-polar lipids such as TAG, cholesterol (Cho) and cholesterol ester are coeluting at retention times close to the void volume of the system, although most phospholipid classes are baseline separated using HILIC method [24]. For total lipid extracts, which include both non-polar and polar lipid classes, the sample should be separated into lipid classes by normal-phase (NP) HPLC. From that point, the target lipid class is separated into molecular species by RP-HPLC [20,21]. The mobile phase solvent used in NP-HPLC may affect the stability of the RP-HPLC stage. This restricts the solvents that may be used. A potential problem relating to 2D-HPLC during the separation is peak broadening. Guo contended that a comprehensive 2D-HPLC method has yet to be perfected [25].

In the present study, we thus constructed an improved online 2D-HPLC system consisting of NP- and RP-HPLC with a high-pressure switching valve, combined with a trapping column and a make-up pump. This 2D-HPLC system can be used with total lipid extracts, including polar lipids and non-polar lipid classes, such as TAGs, fatty acids, ceramide, etc. To identify and quantify the species in a single analysis run, a charged aerosol detector (CAD) and electrospray ionization (ESI)-MS were coupled with our 2D-HPLC system. CAD is a universal HPLC detector and can be used for the detection of lipids with no chromophore, and it can detect impurities that cannot be detected under analysis of targeted compounds by MS [26–29].

This system can analyze the molecular species in one lipid class with a single analysis operation. We used this online 2D-HPLC system to analyze the PE and PC molecular species both in the total lipids extracted from soybean lecithin and in the plasmalogen-rich lipids extracted from chicken breast meat.

2. Materials and methods

2.1. Materials

All the organic solvents used in the HPLC analysis were of the HPLC grade. Acetonitrile and methanol (MeOH) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), while *n*-hexane and methyl *tert*-butyl ether (MTBE) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Ammonium acetate solution (1 mol/L) was purchased from Wako Pure Chemicals (Osaka, Japan). Standard lipids for the NP-HPLC were acquired as follows. Trihexadecanoin (triacylglycerol, TAG) was purchased from Olbracht Serdary Research Laboratories (Toronto, Canada). Hexadecanoic acid (fatty acid, FA) and sphingomyelin from egg yolk were purchased from Nagara Science Inc. (Gifu, Japan). D-α-tocopherol (α-TOH) cholesterol (Cho) and cycloartenyl ferulate (γ-oryzanol, Ory) were purchased from Nacalai Tesque, Inc. Glucosylceramide (GlcCer) was purchased from Matreya Inc. (State College, PA, USA). L-α-phosphatidylinositol ammonium salt solution from bovine liver was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). We acquired 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (lysophosphatidylethanolamine, LPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC) from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine, LPC) was purchased from Apollo Scientific Ltd. (Cheshire, UK). The 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and the 1,2-Distearoyl-*sn*-glycero-3-phosphocholine was purchased from Apollo Scientific, Ltd. (Cheshire, UK). The 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine and 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine were synthesized from L-α-glycerylphosphorylcholine, oleic acid, and linoleic acid purchased from Nacalai Tesque, Inc. (Kyoto, Japan) [30]. The soybean lecithin and purified soybean PCs were donated by J-Oil Mills, Inc. (Tokyo, Japan). As a plasmalogen-rich sample, total lipids were extracted from chicken breast meat purchased from a local grocery store (Fukaishimidzu-cho, Naka-ku, Sakai, Japan).

2.2. Sample preparation

Soybean lecithin (1 mg) was dissolved in a $CHCl_3$/MeOH (2/1, v/v) solution (1 mL, 10 mg/mL). Then, 3-μL aliquots were injected into the 2D-HPLC system for PE and PC analysis. The plasmalogen-rich sample was prepared as follows. Chicken breast meat was cut and freeze-dried in the dark. The freeze-dried sample (8.3 g) was homogenized with a mortar and pestle. The total lipids were extracted by the Folch method [31] and then dried under a gentle stream of N_2 gas. A stock solution of the total lipids was prepared [5 mg/mL in $CHCl_3$/MeOH (2/1, v/v)] and stored at −20 °C in the dark. Then 20- and 10-μL aliquots of the sample solution were injected into the 2D-HPLC system for the PE and PC analysis, respectively.

2.3. 2D-HPLC (NP-HPLC-RP-HPLC/CAD/MS)

An UltiMate 3000 series HPLC system equipped with quaternary pumps, an online degasser, a six-port two-position switching valve, an autosampler, column compartments with thermostats, and variable-wavelength detectors (VWD) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for the NP-HPLC. An UltiMate 3000 series HPLC system equipped with quaternary pumps, an online degasser, a CAD, and an LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for the RP-HPLC/CAD/MS. An LC-10AD pump (Shimadzu Corporation, Kyoto, Japan) was used as a make-up pump. The Chromeleon 6.8 software package (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for system control and data analysis, while the Xcalibur 2.2 software package (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for MS and MS^2 data acquisition and analysis. Fig. 1 shows the configuration of the 2D-HPLC system.

The first-dimensional (1st D)-HPLC (NP-HPLC) separation was performed to separate the targeted phospholipids from other lipid classes in the sample. Prepared samples were loaded onto a YMC-Pack PVA-Sil (250 mm (L) × 4.6 mm (I.D.), 5 μm; YMC Co., Ltd., Kyoto, Japan). The HPLC separation temperature was set to 30 °C and the flow rate was set to 1.0 mL/min. Mobile phases A, B, and C were *n*-hexane, MTBE, and MeOH, respectively. The solvent gradient program was as follows: 0–7 min A/B/C (v/v/v %) 88/10/2; 7–12 min A/B/C (v/v/v %) 2/88/10; 12–22 min A/B/C (v/v/v %) 2/28/70; 22–32 min A/B/C (v/v/v %) 2/28/70; and 32–35 min A/B/C (v/v/v %) 88/10/2. The separation profile was monitored at 210 nm using a VWD.

The trapping column was conditioned with make-up solvent before measuring. The flow rate of the make-up pump was set to 5.0 mL/min and a water/acetonitrile solvent (40/60, v/v %) was used. The temperature of the trapping column was set to 27 °C. When the targeted phospholipid fraction was eluted from the first column, the switching valve was changed from position B to A (Fig. 1). In the trapping line, the targeted phospholipid fraction was mixed with the make-up solvent and trapped in a high carbon content octadecyl-silica (ODS) column (YMC-Pack Pro C18 RS; carbon content: 22%; 30 mm (L) × 4.6 mm (I.D.), 5 μm; YMC Co., Ltd., Kyoto, Japan). The flow channel was switched to a second-dimensional (2^{nd} D)-HPLC (RP-HPLC) system by changing the position of the switching valve (from A to B, Fig. 1). The PE or PC